HOW FDA APPROVED CHYMOSIN: A CASE HISTORY

by Eric L. Flamm

The U.S. Food and Drug Administration (FDA) has accumulated substantial experience with biopharmaceuticals, and now it has some with engineered food ingredients as well: it formally approved the first food ingredient made via recombinant DNA technology just over a year ago (*55 Fed. Reg.* 10932, March 23, 1990). The product is a chymosin enzyme (rennin) preparation derived from *Escherichia coli* K-12 carrying the bovine prochymosin gene. This article details the actual approval process used by FDA.

CHYMOSIN

Chymosin (rennin) is the principal milk-clotting enzyme present in rennet[1,2]. It is a protease that hydrolyzes one bond in the kappa-casein protein of milk, cleaving it into two peptides. Kappa-casein normally stabilizes micelles in milk, but when it is cleaved, the micelles precipitate into curds. After the liquid whey is removed, the curds may be processed into cheese or other dairy products.

Traditionally, most commercial cheeses and other protease-dependent dairy products have been made using rennet isolated from the fourth stomachs of unweaned calves[2]. As consumption of veal fluctuates, so does the availability and price of calf rennet. Rennet was affirmed as GRAS (generally recognized as safe; see box for a detailed explanation of this term) by FDA in 1983 (*48 Fed. Reg.* 51151, Nov. 7, 1983).

Several forms of chymosin are found in calf rennet. The two most predominant and active are chymosin A and chymosin B, isozymes differing by a single amino acid[1,3]. (For the purposes of this discussion, however, they will not be distinguished.)

Calf chymosin is the final expression product of the prochymosin gene, which consists of 9 exons and 8 introns[4]. The primary translation product is inactive due to the presence of a 42 amino acid fragment at its amino terminal end[5]. Acid conditions in the calf stomach and during commercial processing cleave off this fragment, yielding active enzyme[1].

The prochymosin gene has been cloned into several microorganisms, where it produces prochymosin that can be acid-hydrolyzed into active chymosin[6-12]. Researchers have used a variety of techniques to demonstrate that the recombinant chymosin in all cases is functionally equivalent to rennet.

THE FIRST CHYMOSIN PETITION

FDA has received three petitions for chymosin derived from microorganisms, starting in 1987. The first (GRASP 8G0337) requested GRAS affirmation for a chymosin preparation derived from *Escherichia coli* K-12. After reviewing information in the petition and in the published literature, FDA concluded that the principal active component of the chymosin preparation is the same as that of rennet, and that the impurities of the chymosin preparation, which differ from the impurities of rennet, do not render the substance unsafe for its intended use. FDA therefore affirmed this preparation as GRAS for use as a replacement for rennet.

ESTABLISHING IDENTITY

One FDA concern was to determine whether the cloned chymosin is identical to the chymosin in rennet. The petitioners provided published scientific data documenting that the prochymosin gene had been cloned and that it is properly expressed in its microbial hosts to make functional chymosin[6-12].

The petitioners used three lines of evidence to show that the correct gene had been cloned. The cloned DNA was digested with restriction enzymes and the resulting fragments were found to be the sizes predicted by the DNA sequence of the prochymosin gene[7-11]. The cloned DNA, and RNA synthesized from it, hybridized appropriately with the calf prochymosin gene[7-11]. Finally, the sequence of the cloned DNA corresponded to the amino acid sequence of the prochymosin protein[6,7].

The cloned prochymosin gene produced chymosin of the expected size and biological activity. Cloned chymosin has the same molecular weight as chymosin derived from calf rennet, as demonstrated by SDS polyacrylamide electrophoresis[8-14]. Cloned chymosin also has the same functional activity as chymosin derived from calf rennet, as demonstrated by milk clotting assays performed under various conditions of temperature, salt concentration, and pH[8-14].

SAFETY CONCERNS

FDA reviewed the safety of the enzyme preparation by assessing both

Eric L. Flamm is with the Office of Biotechnology, U.S. Food and Drug Administration, HF-6, 5600 Fishers Lane, Rockville, MD 20857. The opinions in this paper are the author's own and do not necessarily reflect those of the Food and Drug Administration.

the enzyme itself and the other components of the preparation. From the data discussed above, FDA concluded that the recombinant enzyme is virtually indistinguishable from the chymosin in rennet. Therefore, FDA concluded that the chymosin enzyme in the microbial preparation is as safe as that of the GRAS calf-rennet preparation.

The other components in the microbial preparation are the impurities derived from the production organism and the materials used in processing. Obviously, these impurities differ from those in a preparation derived from a calf stomach.

The determination that the manufacturing process does not introduce unsafe impurities into the final enzyme preparation was based primarily on the fact that chemicals used in the purification are approved food additives or GRAS substances. Additionally, the purification process destroys the production organism and removes most of the microbial material from the final product.

The isolation of chymosin from *E. coli* K-12, as described in published articles[8,9,13,14] and in the petition, takes advantage of the fact that the prochymosin proteins aggregate into insoluble inclusion bodies when overproduced in *E. coli*. After the cells are grown to the appropriate density in a fermentation vat, they are pelleted, washed, resuspended in a small volume, and lysed. The inclusion bodies are then collected and centrifuged at low speed, leaving in solution the bulk of the cellular material. After washing, the inclusion bodies are solubilized by denaturation in alkaline urea. The prochymosin is then renatured, further purified via anion exchange chromatography, and activated to chymosin via acid hydrolysis.

The production strain used by the petitioner carries an antibiotic resistance marker, the beta lactamase gene encoding ampicillin resistance. To ensure that the food-grade enzyme preparation would not contain functional copies of this gene, the petitioner added one further purification step to those described above—treating the purified inclusion bodies with acid prior to solubilizing them. This step is intended to destroy any unlysed cells pelleted with the inclusion bodies and to degrade any trapped DNA.

The resulting chymosin preparation is significantly purer than traditional rennet. According to data in the petition, some 60-80 percent of the total protein in the preparation is chymosin, as compared with only two percent in commercial rennet. Its use in food processing as a replacement for rennet would result in a very low daily intake, less than a half milligram per person, or 10 micrograms per kilogram body weight.

FDA based its conclusion that the production strain is safe for use in the production of a purified chymosin preparation primarily on published evidence demonstrating that it is nonpathogenic and nontoxigenic. Such evidence includes studies showing that *E. coli* K-12 does not colonize the gut of man or other animals even at high concentrations (10^9-10^{10} viable organisms per ingestion)[15-17], that the K-12 strain has been widely used as a laboratory organism for 30 years with no reported incidents of illness[15], that it does not produce toxins that cause illness upon ingestion[15,16], and that it is deficient in virtually all characteristics necessary for pathogenesis[15]. Additionally, nonpathogenic strains of *E. coli* are a part of the normal flora of the gastro-intestinal tract of man, where they are found at 10^6-10^8 organisms per gram of intestinal content[15].

Thus, even though it is not a common food-use organism and there have been no traditional feeding studies performed with it, FDA concluded that there is sufficient published information on *E. coli* K-12 to demonstrate that it is safe for producing chymosin.

As corroborative evidence of safety of its production strain, the petitioner submitted unpublished data from an *in vitro* test demonstrating that the strain produces no detectable shiga-like toxin, a potent enterotoxin produced by some pathogenic strains of *E. coli*. This was not surprising, since K-12 strains have not been found to contain the shiga-like toxin gene[18].

FDA ON PAPER

While the main article describes the details of FDA's approval of a specific food additive—chymosin—the information in this sidebar explains what FDA expects to see in a product application—and why.

FOOD ADDITIVES AMENDMENT

In 1958, Congress passed the Food Additives Amendment to the Federal Food, Drug, and Cosmetic Act. This amendment, Section 409 of the Act, mandates that food additives be approved by FDA before they may be used in food. Prior to this, FDA had the authority to remove adulterated substances from the food supply, but the burden was on the Agency to demonstrate that the substances were unfit or unsafe for consumption. With this amendment, Congress shifted the burden onto industry to demonstrate that a substance is safe before it may be marketed for use in food.

A food additive is defined, in part, as a substance whose intended use results directly or indirectly in its becoming a component of food or otherwise affecting the characteristics of food (21 USC 321[s]). However, substances that are generally recognized as safe (GRAS) are excluded from the definition. The general recognition of safety must be held by scientific experts, based either on publicly available scientific information or on evidence of common safe use in food prior to January 1, 1958.

A sponsor that wants to market a food additive must, in addition to ensuring that the product is of appropriate food grade, submit a petition to FDA containing information to establish that the substance is safe for its intended use and that it performs its intended function. FDA reviews the information and, if it finds the information adequate, publishes an authorizing regulation listing the substance and the use for which it is approved. Only then can the food additive be legally used in food.

GRAS food ingredients, not being considered food additives, are exempt from the premarket approval requirement. Indeed, many food ingredients that are accepted as GRAS because of their long and widespread use do not appear on any lists maintained by FDA.

However, it is prudent for sponsors to consult with FDA about the regulatory status of new products for which they believe adequate information exists to support a GRAS determination. This is also true for sponsors of traditional ingredients used in significantly new ways or manufactured by significantly new methods. The sponsor otherwise risks challenge from the Agency if it disagrees with his GRAS determination. Sponsors may submit, or may be requested to submit, a GRAS affirmation petition to enable FDA to fully evaluate the GRAS status of such a product.

GRAS affirmation petitions for new (post-1958) or newly made products must contain the same amount of scientific information as required for a food additive petition; additionally, to demonstrate the general recognition, they must document that the information critical to establishing the safety and function of the food ingredient is published. Thus, getting an ingredient affirmed as GRAS—that is, establishing that it is exempt from premarket approval—can be more arduous than obtaining that approval.

Presently, companies that have submitted petitions to FDA for substances produced using the newer methods of biotechnology have all requested that they be affirmed as GRAS. The products are enzymes derived from microorganisms into which DNA has been introduced with recombinant DNA techniques.

SAFETY REVIEW OF BIOTECH PRODUCTS

Before approving the use of a food ingredient, whether produced by traditional or new techniques, FDA must find that a reasonable certainty exists that the product is safe at the levels it is expected to be consumed. When evaluating the safety of microbial enzyme preparations, for example, FDA evaluates the safety of both the enzyme and the impurities likely to be in the preparation. Because the impurities will derive from or be

To further substantiate the safety of the chymosin preparation, the petitioner submitted two unpublished short-term *in vivo* studies—a five-day feeding study in dogs and a one-month gavage study in rats. No adverse effects were observed in these studies at any dose tested. The petitioner also submitted data demonstrating that there was no detectable transformable DNA in the preparation, and no DNA fragments larger than 200 bases detectable by radiolabeled hybridization after gel electrophoresis. For comparison, the coding sequence of the antibiotic resistance gene carried by the production strain is 858 bases[19].

Additionally, the chemical and microbial purity of the enzyme preparation was in compliance with the requirements of the Food Chemicals Codex, 3rd Edition.

CONCLUSIONS

After a comprehensive review of the information in the published literature, FDA concluded that the calf chymosin gene cloned into *E. coli* K-12 is expressed, that the gene product is chemically and biologically indistinguishable from calf chymosin, and that the impurities in the microbial chymosin preparation do not make the preparation unsafe. As for any food ingredient that is new or made by a new method, FDA reviewed the manufacturing method to determine product purity and identity.

From its review of this chymosin preparation and other new biotechnology-derived food ingredients, the Food and Drug Administraton is finding that the review of the safety, purity, and identity of these products is fundamentally no different from that of analogous products derived from unmodified or traditionally modified organisms. It is also finding that biotechnology provides powerful tools for resolving safety questions.

remnants of both the production organism and materials used in the manufacturing process, FDA examines the safety of the production organism, as well as the steps in its fermentation and in isolating, purifying, and stabilizing the final product.

If chemical, microbiological, or molecular biological information shows that the enzyme is the same as or substantially equivalent to an accepted food-use enzyme and that the impurities in the preparation raise no safety concerns then minimum toxicological testing may be necessary.

New substances, however, are likely to require more extensive toxicological testing, the nature of which would be based on considerations such as the identity of the product and the amount in the diet to which consumers would be exposed.

In assessing the safety of the production organism, FDA reviews a variety of factors to determine if the organism is well-characterized for its intended use and if it is pathogenic or toxigenic. For recombinant production organisms, this includes examining all steps in their construction to ensure that all vectors used are safe and that the inserted DNA does not encode unknown or potentially toxic proteins. The entire segment of cloned DNA, including sequences flanking the target gene, should be analyzed. If the donor organism produces toxins or other undesirable compounds, data should be provided demonstrating that DNA encoding these substances was not inadvertently cloned along with the target DNA.

Recombinant microorganisms frequently contain marker genes that encode resistance to therapeutically useful antibiotics. It is FDA's policy that enzyme preparations derived from antibiotic-resistant microorganisms should not contain levels of viable cells, transmissible vectors, or intact DNA that might serve as sources of antibiotic resistance for pathogens with which the preparations come into contact. Therefore, FDA reviews whether there is documentation that such transfer cannot happen at a biologically significant frequency.

ENVIRONMENTAL REVIEW

Under the National Environmental Policy Act (NEPA), FDA and other federal agencies must evaluate the potential environmental effects of their actions and include these evaluations in their decision-making. If the agency concludes that there will be significant effects, it must prepare a detailed environmental impact statement.

To fulfill its NEPA requirements, FDA evaluates whether the manufacture, including the use and disposal of all starting materials, and use of petitioned-for food ingredients could have a significant impact on the environment. For microbially-derived enzymes from recombinant strains, the focus of the environmental review centers around assessing the potential effects of the production organisms at the manufacturing site. The Agency examines many factors, such as whether the production organism has characteristics that would be expected to give it a competitive advantage in the environment; whether it is toxigenic or pathogenic or has other properties that would make it harmful to the environment; whether it contains mobile or easily mobilizeable plasmids or transposons that could transfer traits to other organisms; and the number of viable organisms likely to be incidentally released.

From the extent of its reviews so far, FDA has found that the organisms engineered to produce food-grade enzymes have low potential for causing significant environmental effects. The only characteristic that has raised questions is the presence of antibiotic resistance markers: will resistant production organisms incidentally released at the fermentation plant become established in the environment and become a reservoir for the spread of antibiotic resistance to human or domestic animal pathogens?

Petitioners have not relied on information aimed at directly answering whether the presence of particular antibiotic resistance markers would give host organisms a competitive advantage or would significantly contribute to the spread of antibiotic resistance in pathogens. Rather, the petitioners have focused on showing that the production strains have limited ability to survive and disperse in the environment; that the vectors present are nonconjugative and poorly mobilizeable; and, finally, that little or no release will occur because the production organisms are virtually all destroyed either during processing or prior to disposal from the fermentation plant.

The author would like to thank Henry Miller, Buzz Hoffmann, Zofia Olempska-Beer, Eugene Coleman, Walter Hill, and especially James Maryanski for their comments and suggestions.

REFERENCES

1. Foltmann, B. 1970. Prochymosin and Chymosin (Prorennin and Rennin). Methods in Enzymology **19:**421.
2. Burgess, K. and Shaw, M. 1983. Dairy, pp. 260-265 in *Industrial Enzymology*, T. Godfrey and J. Reichelt (eds.), The Nature Press, New York.
3. Foltmann, B., et al. 1979. The Primary Structure of Calf Chymosin. J. Biol. Chem. **254:**8447.
4. Hidaka, M., et al. 1986. Cloning and Structural Analysis of the Calf Prochymosin Gene. Gene **43:**197.
5. Pederson, V.B. and Foltmann, B. 1975. Amino Acid Sequence of the Peptide Segment Liberated During Activation of Prochymosin. Eur. J. Biochem. **55:**95.
6. Harris, T.J.R., et al. 1982. Molecular Cloning and Nucleotide Sequence of cDNA Coding for Calf Preprochymosin. Nucleic Acids Research **10:**2177.
7. Moir, D., et al. 1982. Molecular Cloning and Characterization of Double-stranded Complementary DNA Coding for Bovine Chymosin EC 3.4.23.4. Gene **19:**127.
8. Emtage, J.S., et al. 1983. Synthesis of Calf Prochymosin (Prorennin) in *Escherichia coli.* PNAS **80:**3671.
9. McCaman, M.T., Andrews W.H., and Files J.G. 1985. Enzymatic Properties and Processing of Bovine Prochymosin synthesized in *Escherichia coli.* Journal of Biotechnology **2:**177.
10. Goff, C.G., et al. 1984. Expression of Calf Prochymosin in *Saccharomyces cerevisiae.* Gene **27:**35.
11. Cullen, D., et al. 1987. Controlled Expression and Secretion of Bovine Chymosin in *Aspergillus nidulans.* Bio/Technology **5:**369.
12. Mellor, J., et al. 1983. Efficient Synthesis of Enzymatically Active Calf Chymosin in *Saccharomyces cerevisiae.* Gene **24:**1.
13. Kawaguchi, Y., et al. 1987. Production of Chymosin in *Escherichia coli* Cells and its Enzymatic Properties. Agricultural and Biological Chemistry **51:**1871.
14. Marston, F.A.O., et al. 1984. Purification of Calf Prochymosin (Prorennin) in *Escherichia coli.* Bio/Technology **2:**800.
15. Gorbach, S.L. 1978. Recombinant DNA: An Infectious Disease Perspective. Journal of Infectious Diseases **137:**615.
16. Curtiss, R. 1978. Biological Containment and Cloning Vector Transmissibility. Journal of Infectious Diseases. **137:**668.
17. Smith, H.W. 1978. Is It Safe to Use *Escherichia coli* K-12 in Recombinant DNA Experiments?" Journal of Infectious Diseases **137:**655.
18. Newland, J.W. and Neill, R.J. 1988. DNA Probes for Shiga-Like Toxins I and II and for Toxin-Converting Bacteriophages. J. Clin. Micr. **26:**1292.
19. Sutcliffe, J.G. 1978. Nucleotide Sequence of the Ampicillin Resistance Gene of *E. coli* Plasmid pBR322. PNAS **75:**3737.